A Method for Producing Cross-linked Hyaluronic acid – Protein Bio-composites

BACKGROUND OF THE INVENTION

1.**Field of Invention**:

This invention relates generally to a new method for producing ~~different types of~~ cross-linked hyaluronic acid – protein bio-composites in various shapes, and in particular, to a method for producing cross-linked hyaluronic acid – protein bio-composites from a homogenous solution ~~that~~ preparing by mixing~~formed by various rates of~~ hyaluronic acid and – protein at various ratios.~~;~~ The prepared bio-composites ~~and~~ can be processed into different sha~~types of the bio-composites~~.

2. **Description of the Related Art**

Hyaluronic acid (HA) is a muco- polysaccharide occurring naturally in and purified from the vertebrate tissues and fluid, and having a linear structure with high molecular weight ~~usually varying within the range of~~ from several thousands to several millions daltons depending on its source and purification method. Karl Meyer et al. in 1934~~HA was~~ first reported ~~by Karl Meyer et al. in 1934,~~ that HA contains glucuronic acid and glucosamine and ~~which~~ was isolated and purified from the vitreous humor of cow. HA is a linear chain polymer having repeat units ~~consists~~ of ~~alternating~~ N-acetyl-D-glucosamine and D-glucuronic acid residues bonded through~~joined by alternating,~~ beta (1→-3)bonding~~glucuronic~~ and then beta (1→-4) bonding. ~~glucosaminidic bonds, so that the repeating unit (dimer) is (1 fwdarw.4) beta-D-GlcA (1.fwdarw.3) beta-D-GlcNAc.~~ HA is widely distributed in connective tissues, mucous tissue, crystalline lens and capsules of some bacteria. In commercial application~~sbility~~, HA has been used as a matrix in drug delivery, an arthritic agent, a healing agent for arthritic operation or general wound healing. In industrial production, HA was mainly extracted and purified from the cockscomb, but HA can also be isolated and produced from the capsules of S*treptococci spp.* ~~*that* produced in a ferment~~ by fermentation bio-technique.

HA aqueous solution shows both a high viscousityness and flexibility. The characteristics of HA that applied in the ophthalmology is generally called named as a viscoelastic matrix when applied in the ophthalmology. These viscoelastic characteristics wereis attributed produced due to the formation of sponge polymeric network formed from bulk molecular volume HA by thehaving high MW and molecular volume of HA. HA is in vivo synthesized byfrom the HA synthetase that exists in the plasma membrane, and hydrolyzed by the hyaluronidase that exists in lysozyme. The interaction of HA and proteoglycans can stabilize the structure of resultant matrix and modify the behavior of cell surface. This characteristic exhibitsprovides many important physiological functions, including: lubrication, water-sorption, water retention, filtration, and modulates the distribution of cytoplasmic protein.

It is knownhas been reported that HA has with many functions of of (1) naturionally occurring in human body, (2) non immune reaction, (3) degradabilitytion and absorbabilityption inby human body, (4) easy availabilitymass production, (5) application in the a high bio-molecular weight bio material applied inof medicine. The major application of HA is in the ophthalmic operation of cataract and cornea damage. High molecular of aqueous HA solution is injected into the eye as a viscoelastic fluid, and plays a special role to maintain the morphology and function of eye. HA has been recently applied in wound healing, tissue anti-adhesion after surgery and drug delivery applications. HA is present in intrabetween cells as a complex with protein in tissue, which forms a jelly matrix owing to it high water retention and can thus be useful infor cosmestic applicationble and play an important role in as an anti-skin aging agent.

Collagen is a structure protein found in animals. It is a naturally occurred bio-polymermolecular, and itscan eliminate moiety causing thean immune-reaction could be eliminated via isolation, purification orand optional treatment with enzyme (such as pepsin), andto giveet collagen having a good bio-compatibility of collagen. Collagen can be processed byvia various reconstruction, chemical cross-linking reactiontechnique and optional additional processing procedure to form into different shapestypes, such as plate, tube, sponge, powder or soft faiberic. Since Ccollagen will

beis a biodegradedable in vivo and is a low toxic polymer having excellent bio-compatibility in thehuman body., It has been used as a hemostatic agent, nerve regeneratieng agent, tissue anaplastic agent, scald dressing material, hernia repair, urethra operation, drug delivery, ophthalmology, vaginal contraceptive, cardiac valve repair, blood vessel operation and operating structure, and other biomedical materials.

Gelatin is a denatured collagen. TheIts amino acid content is similar to the collagen but different in structure and chemic-physical properties are different. Up to date, it has been used in a wide variety of food application and medical research, such as hemostatic cotton and drug delivery.

HA and collagen are the major componentntents of extra-cellular matrix. Gelatin is also made from collagen. Therefore, gelatin is also haswith good bio-compatibility and biodegradation in thumane body. The gelatin composites can be also be used for the development of the implant matrices in the biomedical materials field, such as histological engineering, active ingredient releasinge system of material or as anti-adhesive materials for preventing tissue from sticking after surgery.

(1) Milena Rehakova et al., 1996, Journal of biomedical materials research, vol. 30, pages 369-372,describes thea method for preparing collagen and hyaluronic acid composite materials withrough the use of the glyoxal and starch dialdehyde as a cross-linking agenter. The collagen was dispersed in 0.5M acetic acid solution, and then HA was added to the solution and reacted for 5mins. A fFiber was precipitated was formed and filtered, washed several times with water and alcohol, and dried at a temperature of 35℃, and then a smooth surface of fiber structure in the form of a film having a smooth surface was produced. The cross-linking of the composite material was carriedr out in the presence of an aqueous starch dialdehyde solution;. In the case of using glyoxal as the cross-linking agent, but the cross-linking of glyoxal was carriedr out by adding when HA and glyoxal were added to the suspension of collagen, or addinged glyoxal to the suspension of collagen first and then addinged HA.

(2) Jin-Wen Kuo et al., 1991, Bio-conjugate chemistry, vol,2, pages 232-241, describes a method for preparing water-insoluble derivatives of hyaluronic acid by reacting high molecular HA with the 1-ethyl-3-

(3-dimethylaminopropyl) carbodiimide at a pH of 4.75. In a general experiment, sodium hyaluronate was dissolved in distilled water to produce a 4 mg/ml HA solution. In some reaction, ~~the~~ amine and sodium hyaluronate were added into the HA solution and mixed together. The pH of the aqueous solution was adjusted to pH 4.75. Carbodiimide was dissolved in either water or isopropanol, dependinged on the solubility of carbodiimide.

——After ~~the~~ mixing of HA and carbodiimide, the resultant solution ~~a pH of 4.75~~ was maintained at a pH of 4.75 by addition of 0.1N HCl using a pH meter~~Stat apparatus~~. The reaction mixture was kept at room temperature for 2 hrs, then ~~5% (weight/volume) reaction solution of~~ HCl solution was added until a concentration of HCl was 5% (w/v) in the solution, and then a precipitate is formed after adding 3 time volume solution of ethanol. Non-reacted chemical reagent was washed out for 2-3 times with distilled water. Finally, the precipitate was dissolved in deionozed water before lyophilization.

(3) Lin-Shu Liu et al., 1999, Biomaterials, vol, 20, pages 1097-1108, ~~S~~states a method for preparation of hyaluronate-polyaldehyde by treatment of hyaluronate with sodium periodate. Hyaluronate-polyaldehyde was prepared by oxidizing sodium hyaluronate with sodium periodate. A collagen-hyaluronate matrix was synthesized by ~~the~~ covalent bionding of aldehyde group to the collagen~~,~~ to obtain a material ~~and can be provide to~~for supporting ~~the growth of~~ cartilage tissue or repairing bone ~~repair material~~.

(4) D. Bakos et al., 1999, Biomaterials, vol, 20, pages 191-195, describes a new method for preparing ~~the~~ bio-composite ~~bio~~-material. The composite material consisted of nine parts by weight of inorganic components hydroxyapatite ~~by weight~~ and one part of organic component~~,~~ including 92wt% collagen and 8wt% hyaluronic acid. ~~The fraction of h~~Hydroxapatite particles was gradually added to the solution of hyaluronic acid in de-ionized water, and intensively stirred and mixed. Separately, ~~the dispersion of~~ very fine collagen fibers 1% by dry weight was dispersed in de-ionized water ~~was prepared~~ after dry fibrillation of lyophilized fibers of collagen. The two prepared dispersions were mixed together to form ~~the~~ complex precipitate. The precipitate was filtered and dried at a

temperature of 37□ ~~in PTFE form~~to obtain a composite which did not undergo any cross-linking reaction.

(5) C. J. Doillon et al., 1988, Biomaterials, uses a porous sponge of collagen ~~to~~as a support for the growth of epithelium and fibroblast cell, and as a matrix of artificial skin. HA and/or fibronectin can enhance the repair on~~f~~ wounded skin ~~wound~~ and the proliferation of cell. These high molecular can modify the behavior of cell in tissue culture. The method of preparation ~~was that the~~includes a step of dispersing water-insoluble collagen (1% by weight) ~~was dispersed~~ in hydrochloric acid~~gen chloride~~ solution at a pH 3.0. In this step, 1%w/w of hyaluronic acid, fibronectin, dermatan sulfate and chondroitin-6-sulfate were added to the collagen solution. The dispersion solution was frozen at -30℃,and then lyophilized before cross-linking.

(6) S. Srivastava et al., 1990, Biomaterials, vol, 11, pages 155-161, indicates that collagen gels modified or added with ~~the~~ glucosaminoglycans, e.g.(5% or 10% chondroitin sulfate ~~and~~or less than 5% of HA) ~~on the collagen gels would~~ill enhance the cell growth and adhesion, the growth and adhesion of cells would be inhibited if ~~but~~ more than 5% HA was incorporated into collagen gels ~~inhibited cell adhesion and growth~~.

(7) S. Srivastava et al., 1990, Biomaterials, vol, 11, pages 162-168, studied~~estimated~~ the effect of the collagen or modified collagen on the growth of fibroblast cell line. The preparation of collagen/GAGs and fibronectin composite materials were following ~~as~~ the method described by Yannas ~~described~~. ~~The~~ 3%w/v of degassed collagen slurry was stirred in 0.05M acetic acid ~~solution.~~ while ~~The~~ a solution of HA ~~that~~ dissolved in 0.05M acetic acid was added to the resultant solution until the dry weight of GAGs~~collagen~~ was 2.5% based on the weight of collagen, and then solution was homogenized and degassed. Collagen/HA composite material contains~~mprised~~ 5%, 10%, or 20% GAGs, and collagen/CS composite material contains~~mprised~~ 5%, or 10% chondroitin-4-sulfate and chondroitin-6-sulfate. Their preparation method ~~of preparing~~ was the same as the above described. ~~The~~ 1% ~~f~~Fibronectin was further added to the above composite material, and placed on the petri dish for ~~cell~~ culturinge cell. Experimental results showed that polystyrene was better than nature collagen to be a material of petri dish, but the adhesion of ~~nature~~ collagen was improved by chemical modification or by adding~~ed~~ with~~the~~ fibronectin

and chondroitin-4-sulfate. ~~As~~If ~~the~~ content of HA was more than 5%, however, the cell adhesion and growth of nature collagen matrix could be better than the polystyrene material.

(8) M. Hanthamrongwit et al., 1996, Biomaterials, vol, 17, pages 775-780, studies the effect of the glycosaminoglycans, hyaluronic acid and chondroitin-6-sulfate, diamines and ~~a~~ carbodiimides cross-linking agents on the growth of human epidermal cells ~~o~~in collagen gels. Collagen ge (0.3% w/v) was preparedformed by mixing 4.2 mg/ml collagen solution, a mixture of 10~~X~~times of volume of DMEM and 0.4M NaOH (2:1) , and 1:100 (v/v) acetic acid at a ratio of 7:1:2, and adjusting the solution at pH ~~to~~ 8-8.5 by addition of~~with~~ 1M NaOH. The gels were stood~~allowed to set completely~~ for 2hrs at room temperature. If intend to add GAG, hyaluronic acid and chondroitin-6-sulfate solutions in serum-free DMEM ~~were~~ substituted~~prepared at 3mg/ml in 1X serum-free DMEM and incorporated into the collagen solution at various percentages by replacing the 2 volumes of~~ for acetic acid used in the above solution at various ratio. After forming gels, 1-ethyl-3- (3-dim hyaluronic acid and chondroitin-6-sulfate ethylaminopropyl carbodiimide) and diamine were incorporated into the gels ~~was used as a~~to subject to cross-linking ~~agent~~reaction.

(9) L.H.H. Olde Damink et al., 1996, Biomaterials, vol, 17, pages 765-773, describes that ~~treats the cross-linking of~~ non-cross-linked ~~DSC~~ ~~(~~dermal sheep collagen, ~~)~~(N-DSC) was cross-linked with EDC to give E-DSC ~~was performed~~ by immersing 1g N-DSC samples ~~weighting 1g~~ (1.2mmol ~~carboxylic acid groups~~) in 100ml of an aqueous solution containing 1.15g (6.0mmol) EDC at room temperature for 18hrs. During the reaction, a pH of the solution ~~5.5~~ was maintained at 5.5 by addition of 0.1M HCl using a pH meter~~Stat apparatus~~. The molar ~~amount~~ of ~~carboxylic acid group (COOH)of~~ N-DSC samples was calculated assuming that 120 carboxylic acid group ~~containing~~ residues are present per α-chain ($\simeq$1000 amino acids) and that eachα-chain has a molecular weight of 100,000. After cross-linking, E-DSC samples were washed for 2hrs in a 0.1M Na_2HPO_4 solution and subsequently washed four times with distilled water before lyophilization. The other cross-linking of N-DSC with EDC and NHS to give E/N-DSC was performed by immersing N-DSC samples in

aqueous solution containing EDC and NHS at room temperature for 4hrs. The results showed that addition of N-hydroxylsuccinimide to the EDC-containing cross-linking solution (E/N-DSC) increased the rate of cross-linking.

(10) Yannas et al., 1997, U.S. Pat. No. 4,060,081, states a multilayer membrane which is useful as synthetic skin. Preferred materials for the first layer are cross-linked composites of collagen and a muco-polysaccharide. A second layer is formed from a nontoxic material which controls the moisture flux of the overall membrane.

(11) Yannas et al., 1981, U.S. Pat. No. 4,280,954, states a method for preparing cross-linked collagen-muco-polysaccharide composite materials. A collagen solution at pH 3.2 and muco-polysaccharide solution (weight ratio is 6%-15% by weight) were mixed together, and then a precipitate of aldehyde covalent cross-linked collagen-muco-polysaccharide composite was formed.

(12) Yannas et al., 1982, U.S. Pat. No. 4,350,629 discovers that if collagen fibrils in an aqueous acidic solution (< pH 6.0) are contacted with a cross-linking agent (glutaraldehyde) before being contacted with glycosaminoglycan, the materials produced have extremely low level of thrombogenicity. Such materials are well suited for in-dwelling catheters, blood vessel grafts, and other devices that are in continuous contact with blood for long periods of time.

(13) Yannas et al., 1984, U.S. Pat. No. 4,448,718, describes a process for preparing a cross-linked collagen- glycosaminoglycan composite material which comprises forming an uncross-linked composite material from collagen and a glycosaminoglycan and containing the uncross-linked composite with a gaseous aldehyde until a cross-linked product having an M. sub. C of from about 800 to about 60,000 is formed.

(14) Balazs et al., 1986, U.S. Pat. No. 4,582,865, states a method for preparing cross-linked gels of hyaluronic acid and products containing such gels. The cross-linking HA or HA/hydrophilic polymers (polysaccharide or protein) and the divinyl sulfone was carried out at 20°C. in a pH>9 solution. In the 1%-8% dry solids content of mixture, HA contains 5%-95% of dry solids content.

(15) Liu et al., 1999, U.S. Pat. No. 5,866,165, states a matrix and a method for preparing it, which matrix ~~are~~is provided to support the growth of bone or cartilage tissue. A polysaccharide is reacted with an oxidizing agent to open sugar rings on the polysaccharide to form aldehyde groups. The aldehyde groups are reacted to form covalent linkages to collagen. Collagen and polysaccharide used to form matrix are present in a range of 99:1 to 1:99 by weight, respectively. 1% to 50% of the repeat units in polysaccharide are oxidized to contain aldehyde groups.

(16) Pitaru et al., 1999, U.S. Pat. No. 5,955,438, states a method for producing a collagen matrix which may be formed into a membrane useful in guided tissue regeneration. A collagen matrix comprises collagen fibrils ~~which~~ are incubated with pepsin in a solvent, and are then cross-linked to one another by a reducing sugar. Finally, the matrix is subjected to critical point drying.

(17) Pierschbacher et al., 1999, U.S. Pat. No. 5,955,578, states a method for producing polypeptide-polymer conjugates active in wound healing. A synthetic polypeptide comprising the amino acid sequence dArg-Gly-Asp is bonded to a biodegradable polymer via a glutaraldehyde cross-linking agent. The purpose of synthetic matrix is to promote cell attachment and migration.

(18) Hall et al., 1998, U.S. Pat. No. 5,800,811, states a method for producing an artificial skin. An artificial skin is prepared by impregnating a collagen with a transforming growth factor-beta, and incubating the impregnated matrix with a source of stem cells.

(19) Stone et al., 1989, U.S. Pat. No. 5,880,429, states a method for producing a prosthetic meniscus. A pore size in the range 10-50 microns of prosthetic meniscus is formed by type I collagen fibrils 65%-98% by dry weight and glycosaminoglycan molecular (chondroitin-4-sulfate; chondroitin-6-sulfate; dermatan sulfate or hyaluronic acid; 1%-25% by dry weight) and which is adapted for in growth of meniscal fibrochondrocytes.

(20) Stone, 1992, U.S. Pat. No. 5,108,438, states a method for producing a prosthetic inter-vertebral disc. The disc includes a dry, porous, volume matrix of bio-compatible and bio-~~r~~degrad~~sorb~~able fibers which may be interspersed with glycosaminoglycan molecules (0-25% by dry weight).

The cross-linking agent is selected from the group consisting of glutaraldehyde, carbodiimides and so on.

(21) Silver et al., 1987, U.S. Pat. No. 4, 703,108, states a method for preparing biodegradable collagen-based matrix in sponge or sheet form. HA and collagen are added to a dilute HCl solution of pH 3.0 and the mixture is homogenized in a blender. The solution is then poured into a vacuum flask and de-aerated at a vacuum, and then cross-linked with carbodiimide. After then, the matrix is allowed to air dry or freeze dry. The product of collagen-based matrix is cross-linked by immersion in an aqueous solution containing 1% by weight of cyanamide at pH 5.5 for a period of 24hrs at 22℃. HereinaAfter ~~removal~~, the matrix is ~~washed in several changes of water over 24hrs,~~frozen and ~~freeze~~ dried at -65℃ in a vacuum.

(22) Silver et al., 1990, U.S. Pat. No. 4, 970,298, states a porous biodegradable collagen sponge-like matrix ~~having a pore size and morphology~~ which enhances the healing of ~~a~~ wound. Collagen is dispersed in an acid solution ~~HCl at~~of pH from 3.0 to 4.0 and ~~is added to~~mixed with the fibronectin in an acid solution of pH 3.0 to 4.0 ~~and the mixture is dispersed~~ in a blender. Collagen dispersions to be converted into sponge are frozen at -100℃ before freeze drying at -65℃. The matrix is cross-linked in~~by~~ two ~~cross-linking~~ steps consisting of first cross-linking with carbodiimide and then subjecting to~~before~~ dehydrothermal, or first subjecting to~~cross-linked with carbodiimide after~~ dehydrothermal and then cross-linking with carbodiimide.

SUMMARY OF THE INVENTION

~~To b~~Based on the reports of patents and references above-mentioned, the general preparation of the polysaccharide-protein bio-composites is under ~~the~~an acid condition, a polysaccharide-protein fiber precipitate is formed by ~~the~~ forming ionic bond ~~formation~~ between polysaccharide and protein from ~~of the~~ mixing~~ture of little~~minor amount of polysaccharide (less than ~~the~~ 15% weight of collagen) and protein, and then resultant precipitate is further cross-linked ~~form the covalent bond~~ with ~~the~~a cross-linking reagent to form the covalent bond, a non-directional fiber sponge or porous

matrix is produced after washing, filtration and lyophilization. ~~The~~A defect of this process~~dure~~ is ~~just can~~ only that ~~produce~~ a non-homogeneous porous matrix ~~of~~having fiber structure ~~and non-homogeneous composite~~, other than a homogenous composite, can be produced, it is difficult to form ~~the different types of composites it depends on the need of~~ impalpable matrices in a suitable shape as desired. If a shape is required, ~~In a general experiment,~~ a piece of the prepared precipitate ~~was~~is generally homogenized by chopping it into ~~many~~ small segments, and the homogenized slurry was then poured into ~~the different shaped~~ a mold having the desired shape~~that the experiment wanted~~, then lyophilized. According to~~The~~ method of~~developmental techniques of~~ the~~is~~ present invention, ~~can prepare the different ratio of the~~ a mixture solution consisting of polysaccharides and protein at various ratio having, ~~the~~ different pH value is prepared ~~of the homogeneous polysaccharide-protein solution~~, and then ~~can be~~ processed into ~~different types of the~~ bio-composites having different shapes as desired (such as membrane, sponge, fiber, tube or micro-granular and so on). ~~After~~Subsequently, the bio-composites is subjected to a ~~thorough~~ cross-linking reaction ~~within~~ a solution of ~~the~~ water~~/~~ and organic solvent~~ution,~~ to obtain impalpable bio-composites which is a homogeneous, ~~good~~ bio-compatible, biodegradable~~ed~~ and has~~,~~ excellent physical properties and a prolonged enzymatic degradation time~~and fine physics of impalpable bio-composite is formed~~.

The advantage of this invention is that ~~the~~a homogenous polysaccharide-protein solution can be prepared under a wide range of pH value, not only under~~in~~ ~~the~~an acid condition, and the weight ratio of polysaccharide to protein is from 2/98 to 90/10. In ~~a~~ traditional methods~~experiment~~, the collagen is usually used as a major component~~material~~ and the polysaccharide is used as an additive, the maximal ratio of polysaccharide to collagen is around 20%. Besides, the matrix solution ~~that~~ produced from this invention ~~is~~possesses ~~with~~ a uniform~~homogeneous~~ density and a porosity, and can be manufactured into various shape~~type~~s, including ~~the shape of~~ membrane, sponge, fiber, tube or fine particles~~micro-granular~~ and so on. It can also avoid the loss of polysaccharide and reduce ~~the~~ reaction time to only 2-4hrs if ~~while react~~

~~the~~a cross-linking reaction with carbodiimide is conducted in the presence of ~~the little~~weak acid in~~of~~ organic solventsution.

In prior arts~~most cases of the previous study~~, it usually uses ~~the~~ aldehydes ~~was usually~~ as a cross-linking reagent~~,~~. If~~but when used the~~ carbodiimide is used as a cross-linking agent~~reagent~~, the cross-linking reaction ~~wa~~is always conducted~~finished~~ in ~~the~~ water and will take place ~~the reaction time may need~~ for more than 24hrs.

There are many advantages in this invention. ~~and t~~The ~~developmental~~ techniques of the~~is~~ present invention ~~are also never~~ have~~ve~~ never been described in previous references~~study~~. According to the method of the present invention, ~~Therefore, the different shapes of the cross-linking polysaccharide-protein~~ bio-composites which can be produced in various shape ~~contain highly commercial apply and~~ are suitable for using in a ~~wide~~ variety of fields, including biomedical, materials engineering, histological engineering, medical equipment, pharmacy and cosmetic ~~uses~~fields.

Other features and advantages of the present invention will be apparent from the following description of the preferred embodiments thereof~~,~~ and ~~from~~ the appended claims.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The~~is~~ present invention relates to a ~~new~~ method for producing ~~different types of cross-linking~~ polysaccharide-protein bio-composites having various shapes. The advantage of the ~~characteristic of this~~ method~~–~~ is that the~~the various ratio of the homogeneous the polysaccharide material~~ bio-composite can be produced into various shapes, such as membrane, sponge, fiber, tube, and micro-granular. After further subjecting to cross-linking reaction, a bio-composite which is bio-compatible, biodegradable~~ed~~, non-toxic, and impalpable, and possesses prolonged enzymatic degradation time~~,~~ and excellent ~~good~~ mechanical strength~~,~~ ~~on toxicity and impalpable bio-composite~~ is formed. It is extremely~~highly~~ suitable for the application in~~of~~ biomedicine, histological engineering, materials engineering, medical equipment and cosmetic fields. The ~~use of the~~ bio-composite prepared by the present method is suitably used ~~includ ing~~

as ~~to~~ hemosats, vascular sealants, orthopedic implant coatings, vascular implant coatings, dental implants, wound dressings, anti-adhesion barriers, platelet analyzer reagents, research reagents, engineering of cartilage, artificial tendons, blood vessels, nerve regeneration, cornea implants, cell preserving~~ation~~ solutions and for delivering growth factor and/or drugs ~~delivery~~. According to the ~~practice of this~~ present invention, the prepared bio-composite can be further processed into various products possessing~~the~~ high~~ly~~ additional value ~~of by products can be produced~~. It is very useful for commercial utilization.

The~~is~~ present invention ~~is~~ relate~~sd~~ to~~with~~ a ~~new~~ method for producing polysaccharide-protein bio-composites, comprising the steps of:

(a) preparing a polysaccharide aqueous solution;~~.~~

(b) preparing a protein aqueous solution;~~.~~

(c) ~~adjusting the pH and salt content of~~ well-mixing the solutions from steps (a) and (b) , adjusting a pH of the mixture into a moderate range~~s~~ by adding either an acid or a hydroxide, ~~and then according to the design of experiment, the well mixed solution can be prepared~~ processing the mixture into matrix having a desired ~~different~~ shape~~s~~, such as membrane, porosity, sponge, tube or micro-granular and so on;~~.~~

(d) subjecting ~~immerging~~ the matrix to cross-linking reaction in ~~the~~a mixture of water~~/~~ and organic solvent~~sution~~ ~~that~~ containsing ~~a~~the cross-linking~~ed~~ reagent~~, and reacting~~ under ~~the moderate~~a pH of from 4 to 4.5 a~~n~~d a temperature of from 20 to 45℃ for a period of 1 to 6 hours, preferably 2 to 4 hours;~~.~~

(e) washing the matrix for several times ~~selectively~~, and immersing in ~~the~~a salt aqueous solution which~~that~~ is chosen from the group consisting of sodium chloride, dibasic sodium phosphate or ~~the~~a mixture thereof ~~both~~.

The matrix was then further washed several times with large volumes of de-ionized water and lyophilized.

~~As~~In the Step ~~above~~ (a) ~~described~~, the polysaccharide is chosen from the group consisting of hyaluronic acid, carboxymethyl cellulose, pectin, starch, chondroitin-4-sulfate, chondroitin-6-sulfate, alginate, chitosan, agar, carragenan and guar gum, and a mixture thereof.

~~As above~~In the step (b) ~~described~~, the protein ~~solution~~ is chosen from the group consisting of collagen, gelatin, or ~~the~~a mixture thereof ~~both~~.

~~As above~~In the step (c) ~~described~~, the preferred pH value is in a range between 3 and 11,and ~~the change of~~if an intended pH is less than 7, it is~~was~~ adjusted by adding ~~with the~~ acetic acid, hydrochloric acid~~gen chloride~~, or a mixture thereof. If an intended pH is more than 7, it is adjusted by adding sodium hydroxide, potassium hydroxide, or a mixture thereof~~, or the mixture of both that can donate the proton group of acid or the hydroxyl group of alkalinity~~.

The ~~total dry~~ solids content of resultant polysaccharide-protein mixture ~~solution~~ is in a range between 0.2% and 4.0% by weight, ~~but~~and the ~~weight~~ percent of polysaccharide is in a range between 2% and 98%, based on the total weight of the mixture. ~~The concentration of salt is in a range between 0.05M and 0.25M that depends on the selection of the various kinds of acid and hydroxyl compounds.~~

~~As above (c) described,~~As to the procedures ~~of~~for forming the matrix into ~~the~~ different shapes in the step (c)~~of the material~~ are illustrated in details as ~~the~~ follows~~ing~~:

(1) The ~~film~~ matrix is prepared as a film matrix ~~formed~~ by casting the degassed matrix~~ixture~~ consisting of polysaccharide and protein solutions into a mold and ~~allows to~~ drying in an oven at a temperature of 35 to yield a film matrix ~~in an oven at 35℃~~.

(2) The ~~porosity~~ matrix is prepared as a porous matrix~~formed~~ by casting the degassed matrix~~ixture~~ consisting of polysaccharide and protein solutions into a mold in a refrigerator at a temperature of -80℃ and drying at a ~~allows to~~ vacuum ~~dry~~ to yield a porous matrix having a inter-connective porous structure ~~in a freeze dry drying, the porosity of matrix is in the form of a pore morphology with the interconnectivity structure~~.

(3) The ~~powder~~ matrix is prepared as a powder matrix ~~formed~~ by dropping the degassed matrix~~ixture~~ consisting of polysaccharide and protein solutions into the freezing solution at a temperature of -80℃ by using~~with a moderate size of the~~ a syringe, and lyophilizing under a~~allows to~~ vacuum ~~dry~~ to yield a powder matrix ~~in a freeze dry drying~~.

(4) The ~~fiber~~ matrix is prepared as a fiber matrix~~formed~~ by squeezing the degassed matrix~~ixture~~ consisting of polysaccharide and protein solutions into ~~the~~a coagulant solution in~~of~~ a mixture of water and organic solvents ~~with the squeezer apparatus~~, and lyophilizing~~allows to dry~~ to yield a fibrous matrix having a thickness of from 50um- to 1mm ~~thickness~~.

~~As above described, the coagulant solution comprises water and organic solvent.~~ The organic solvent contained in the coagulant solution is chosen from the group consisting of 1,4-dioxane, chloroform, methylen~~e~~ chloride, N,N-dimethylformamide, N,N-dimethylacetamide, ethyl acetate, acetone, methyl ethyl ketone, methanol, ethanol, propanol, isopropanol, butanol, and ~~the~~a mixture thereof ~~each organic solvent~~ the percentage~~weight fraction~~ of the organic solvent in the coagulant solution is between 60% and 100% by weight, ~~but the~~ preferably~~red weight fraction of organic solvent is~~ between 75% and 100% by weight, ~~but the preferred weight fraction of organic solvent is between 75% and 100%~~. The preferred organic solvent is a mixture of ketones and alcohols ~~can be mixed with any ratio~~.

~~As above step (d) described, the preferred~~ The cross-linking agent in step (d) is preferably ~~the~~a carbodiimide, ~~and the carbodiimide~~which is selected from the group consisting of 1-methyl-3-(3-dimethylaminopropyl)-carbodiimide, 3-(3——dimethylaminopropyl)-3-ethyl——carbodiimide, 1-ethyl-3-(3—dimethylaminopropyl)—carbodiimide or ~~the~~any mixture thereof ~~each group~~.

~~As above step (d) described, t~~The mixture of water/ and organic solution in the step (d) is preferably consisting of~~contains~~ 5%-50% by weight of water and~~of~~ 95-50% by weight of either ethanol or acetone, or the both ~~solution~~, ~~but the~~ preferably~~red~~ consisting~~tent is~~ of 5%-30% by weight of water and 95-70% by weight of either ethanol or acetone, or the both. ~~The pH of mixture is in a range between 4 and 5.5, the temperature of reaction is at 20‥ 45‥, the reaction time is 1-6hrs, but the preferred time is in a range between 2-4hrs.~~

~~As above step (e) described, t~~The salt aqueous solution in step (e) is used at a concentration of ~~immersed concentration of salt is in a range between~~ 0.15-4M. ~~solution that consists of sodium chloride, dibasic sodium~~

~~phosphate or the mixture of both.~~ The immersion~~ed~~ time is in a range between 30mins and 3hrs.

The present invention is described in more detail in the following example. These examples are giving by way of illustration and are not intended to limit the invention except as set forth in the claims.

~~The preferred for producing different shapes of polysaccharide protein bio-composites is described in more detail in the following samples.~~

Example 1A-1G: ~~The p~~Preparation of hyaluronic acid/collagen matrix

Hyaluronic acid (HA) (60mg) and collagen (40mg) were each dissolved ~~in~~ in~~the~~ different solvent as shown~~conditions, respectively (as~~ in table 1 described), and then the ~~two~~ prepared two solutions were mixed together to form a mixture that ~~the~~a weight ratio of HA to collagen is 3 to 2 and ~~the total dry~~a solid content of the mixture is 1%.

The resulting ~~solution~~mixture was cast into a mold made of Teflon to yield ~~cross-linked~~ a film. The films prepared in Example~~Sample~~ 1D and 1E had the optimal morphology and physics properties~~after cross-linking~~.

Table 1

~~S~~Example	1A	1B	1C	1D	1E	1F	1G
HA[a] solvent	H_2O	0.1N NaCl	0.1M CH_3COOH	H_2O	H_2O	H_2O	H_2O
Collagen	0.5M CH_3COOH	0.1M CH_3COOH	0.1N NaCl	0.1M CH_3COOH	~~After~~ dDissolvinged in 0.5M acetic acid, Then adjusting pH by 1N NaOH	Dissolving in water and then adjusting pH 7 by HCl	A mixture of 0.5M CH_3COOH and 1N NaOH-~~mixture solution~~
NaCl	-	-	-	30mg	-	-	-
mixed solution	white fiber precipitate	transparence and low viscosity	transparence	transparence	transparence	fine fiber precipitate	white fiber precipitate

1N NaCl	few drops, fiber precipitate and then dissolved	-	-	-	-	-	-
PH	~9	~8	~7	~3	~6	~7	~6
morphology	fine fiber on the matrix surface	semi transparence	semi transparence	white and densecity	White, densecity and high toughness	fine fiber on the matrix surface	white

Example 2: ~~The p~~Preparation of HA/gelatin matrix

HA (50mg) was dissolved in 5ml of pure water. ~~s~~Separately, ~~odium chloride (30mg) was gradually added to the solution of~~ gelatin (50mg) was dissolved in 5ml of warm water (more than 55℃) and then added with sodium chloride (30mg).

The ~~two~~ prepared two solutions were mixed together to form a 10ml mixture of which~~that the~~ pH ~~of solution~~ is around 6.5, the weight ratio of HA to collagen is 1 to 1 and ~~athe total dry~~ solids content is 1%.

The resulting solution was cast into a mold made of Teflon ~~plate~~ and allowed to dry in~~under~~ an oven to yield a transparent film.

Example 3: ~~The p~~Preparation of ~~different salt concentration of~~ HA/collagen matrix at different salt concentration after neutralization.

HA (60mg) was dissolved in pure water. Separately, ~~C~~collagen (40mg) was dissolved in 0.5M acetic acid solution, and then neutralized with sodium hydroxide. Adjust the salt concentration ~~after~~of neutralization and maintain the pH ~~to~~at 6 by ~~change~~adding ~~the~~various volume ~~ratio~~ of water, acetic acid and sodium hydroxide ~~(~~ as shown in ~~t~~Table 2 ~~described)~~. The ~~two~~ prepared two solutions were mixed together to form a 10ml mixture in which~~that the~~ a weight ratio of HA to collagen is 3 to 2 and ~~the total drya~~ solids content is 1%.

The resulting solution was cast into a mold made of Teflon ~~plate~~ and allowed to dry ~~under~~in an oven to yield a film.

Table 2

~~S~~Example	~~2~~3A	~~3~~2B	~~2~~3C
H_2O (ml)	5.5	7.0	8.5
0.5M CH_3COOH	3.0	2.0	1.0
1N NaCl	1.5	1.0	0.5
Salt conc of neutralization. (M)	0.15	0.1	0.05

Example 4: ~~The p~~Preparation of ~~the different pH of~~ HA/collagen matrix at different pH

HA (60mg) was dissolved in pure water. Separately, ~~C~~collagen (40mg) was dissolved in 0.5M acetic acid solution, and then neutralized with sodium hydroxide. Adjust ~~the salt concentration of neutralization to 0.15M and the~~a pH value by adding various volume of acetic acid and sodium hydroxide ~~(~~ as shown in ~~t~~Table 3 ~~described)~~ and maintain a salt concentration after neutralization at 0.15M. The ~~two~~ prepared two solutions were mixed together to form a 10ml mixture in which~~that~~ ~~the~~a weight ratio of HA to collagen is 3 to 2 and ~~the total dry~~a solids content is 1%.

The resulting solution was cast into a mold made of Teflon ~~plate~~ and allowed to dry in an~~under~~ oven to yield a transparent film.

Table 3

~~S~~Example	~~3~~4A	~~3~~4B	~~3~~4C
H_2O (ml)	3.5	5.5	5.44
0.5M CH_3COOH	5.0	3.0	3.0
1N NaCl (ml)	1.5	1.5	1.56
PH value	4.7	6.0	11.0

Example 5: ~~The p~~Preparation of ~~the different ratio of~~ HA/collagen

matrix at different ratio

HA was dissolved in pure water. Separately, Ccollagen was dissolved in 0.5M acetic acid solution, and then neutralized with sodium hydroxide. Adjust theA salt concentration afterof neutralization is maintained atto 0.15M, and thea pH is maintained atto 4.7, and by changing the volume ratio of added water, acetic acid and sodium hydroxide is maintained at 3.5:5:1.5. The two prepared two solutions were mixed together to form a 10ml mixture in whichthat the a weight ratio of HA to collagen is as showndescribed asin tTable 4 and the total drya solids content is 1%.

The resulting solution was cast into a mold made of Teflon plate and allowed to dry in anunder oven to yield a transparent film.

Table 4

SExample	45A	45B	45C	45D	45E	45F
HA (mg)	90	80	60	50	20	2
Collagen (mg)	10	20	40	50	80	98
Weight ratio (HA/collagen)	9:1	4:1	3:2	1:1	1:4	1:49

Example 6: The pPreparation of the different total dry solids content of HA/collagen matrix at different solid content

HA was dissolved in pure water. Separately, Ccollagen was dissolved in 0.5M acetic acid solution, and then neutralized with sodium hydroxide. MaintainAdjust thea salt concentration afterof neutralization toat 0.15M and thea pH toat 4.7, by changing the volume ratio of added water, acetic acid and sodium hydroxide is at 3.5:5:1.5. The two prepared two solutions were mixed together to form a 10ml mixture in which that thea weight ratio of HA to collagen is 3 to 2 and the total drya solids content is as shown

indescribed as tTable 5.

The resulting solution was cast into a mold made of Teflon plate and allowed to dry under oven to yield a transparent film.

Table 5

SExample	56A	56B	56C
HA (mg)	120	60	30
Collagen (mg)	80	40	20
Solid content %	2	1	0.5

Example 7: The pPreparation of the fiber HA/collagen matrix in a fiber form

HA (100mg) was dissolved in 3.5ml of pure water. Separately, Ccollagen (100mg) was dissolved in 5ml of 0.5M acetic acid solution, and then neutralized with 1.5ml of 1N sodium hydroxide. The salt concentration of neutralization is 0.15M. The two prepared two solutions were mixed together to form a mixture in which that thea pH of solution is around 4.7, thea weight ratio of HA to collagen is 1 to 1 and the total dry a solids content is 2%.

The resulting solution was continuouslyal pressed into thea 95% alcohol solutionvent to form a mono-filament fiber by using the different size of syringes having various sizes, and allowed to dry underin an oven to yield a HA-protein matrix.

Example 8: The pPreparation of the micro-granular HA/collagen matrix in a form of micro-granular

HA (100mg) was dissolved in 3.5ml of pure water. Separately, Ccollagen (100mg) was dissolved in 5ml of 0.5M acetic acid solution, and then neutralized with 1.5ml of 1N sodium hydroxide. The salt concentration

afterof neutralization is 0.15M. The two prepared two solutions were mixed together to form a mixture in which that thea pH of the mixturesolution is around 4.7, thea weight ratio of HA to collagen is 1 to 1 and athe total dry solids content is 2%.

The micro-granular matrix was formed by dropping the resulting mixturesolution into the liquid Nnitrogen and lyophilized.

Example 9: The pPreparation of the porous HA/collagen matrix in a porous form

HA (100mg) was dissolved in 3.5ml of pure water. Separately, Ccollagen (100mg) was dissolved in 5ml of 0.5M acetic acid solution, and then neutralized with 1.5ml of 1N sodium hydroxide. The salt concentration afterof neutralization is 0.15M. The two prepared two solutions were mixed together to form a mixture in whichthat the a pH of solution is around 4.7, thea weight ratio of HA to collagen is 1 to 1 and the total drya solids content is 2%.

The resulting solution was cast into a mold made of Teflon plate at a temperature -80℃ and allowed to dry to yield a porous sponge matrix after lyophilization.

Example 10: The effect of cross-linked agent on the chemical cross-linking reaction of HA/collagen matrix.

The film of sExample 56A was chopped to equal pieces in equal size and immersed in the EDC to subject to cross-linking reaction for 2hrs at 30℃ for 2 hours (experimental conditions were shown inas tTable 6 described). The mixtureatrix was then washed 3 times with 80% acetone aqueous solution, each washing time is 20mins. After then, the mixtureatrix was further washed 3 times with de-ionized water, each washing time is also 20mins. Finally, the mixtureatrix was spread on a substrate and dried. The cross-linked film was subject to swelling test by immersinged in 0.15M sodium chloride solution for swelling test, incubatinged for 5 days with gentle shaking at 37℃, then the swelling behavior was observed. From the results shown in Table 6, it results showed that in order to avoid the dissolution of matrix and enhance the cross-linking efficiency, the

cross-linking of matrix was only carried out in ~~the~~a mixture of water/ and organic solvent~~ution that contained the cross-linked agent~~ (sExamples ~~6~~10D,~~6~~10E).

Table 6

~~S~~Example	~~6~~10A	~~6~~10B	~~6~~10C	~~6~~10D	~~6~~10E
EDC conc. (wt%)	2.3	2.3	2.3	2.3	2.3
Solvent	H_2O	PH4.7 solution	PH4.8 solution	80% ethanol	80% acetone
Morphology	thinness	thinness	thinness	normal	normal
Dissolveing test	Soluble	soluble	soluble	insoluble	insoluble

Example 11: The effect of a concentration of cross-linked agent on the ~~chemical~~ cross-linking reaction of HA/collagen matrix.

The film of sExample ~~5~~6A was chopped to ~~equal~~ pieces in equal size and immersed in 80% acetone solution containing EDC at pH 4.7~~, that contained EDC~~ and at 30℃ for 2hrs ~~at 30℃~~ (experimental conditions were shown in~~as~~ tTable 7 ~~described~~). The mixture~~atrix~~ was then washed 3 times with 80% acetone solution, each washing time is 20mins. After then, the mixture~~atrix~~ was further washed 3 times with de-ionized water, each washing time is also 20mins. Finally, the mixture~~atrix~~ was spread on a substrate and dried. The cross-linked film was subjected to swelling test by immersinge~~d~~ in 0.15M sodium chloride solution ~~for swelling test~~, incubatinge~~d~~ for 5 days with gentle shaking at 37℃, then the swelling behavior was observed. Hyaluronidase (220U/ml) was dissolved in 0.15M sodium chloride. Film was weighted and put~~laced~~ into the enzyme solution for ~~the~~ testing ~~of~~ enzyme degradability of the film~~tion~~. After 24 hours, ~~T~~the solution was taken out ~~after 24hrs~~ for uronic acid assay, and then the percent of hydrolysis of HA film was calculated. From the results in Table 7, it ~~results~~ showed that the rate of enzyme degradation of the

cross-linked film ~~which~~ ~~p~~prepared~~ocedure~~ by the~~is~~ present method was reduce~~dible~~ significantly.

Table 7

~~S~~Example	11~~7~~A	~~7~~11B	~~7~~11C	~~7~~11D	Control
EDC (wt%)	0.625	1.25	2.5	5	-
Dissolveing test	insoluble	insolubl e	insolubl e	insoluble	soluble
HA enzyme degradation (%)	1.87	1.5	0.68	1.02	31.13

Example 12: ~~The chemical c~~Cross-linking reaction of ~~the~~a porous HA/collagen sponge matrix~~1~~.

The porous sponge of ~~s~~Example 9 was placed in an oven at 110□ and under a vacuume~~d~~ for 3hrs ~~at 110℃~~. The ~~D~~dried specimens wa~~eres~~ then immersed in ~~the~~a 80% acetone solution for 30mins, and then transferred to a 80% acetone solution containing 2.5% EDC at pH 4.7 ~~that contained 2.5% EDC~~.

The specimens wa~~eres~~ taken out after ~~2hrs~~ reaction at 30℃ for 2 hours, and then washed 3 times with 80% acetone, each washing time is 20mins. After then, the specimens were~~as~~ further immersed in 1M sodium chloride for 20mins, and washed 3 times with deinoized water, each washing time is also 20mins. Finally, the specimens were~~as~~ spread on a substrate and dried.

Example 13: Determination of ability of growth of cell and cyto-toxicity of ~~The~~ cross-linke~~ding~~ HA/collagen ~~for the cell growth and cyto-toxicity~~.

The films prepared from ~~s~~Examples 45C, 45D and 45E were immersed in the 80% acetone solution containing 2.5% EDC at pH 4.7 ~~that contained 2.5% EDC~~. The film was taken out after ~~2hrs~~ reaction at 30℃ for 2 hours, and then washed 3 times with 80% acetone, each washing time is 20mins.

After then, the film was further immersed in 1M sodium chloride for 20mins, and washed 3 times with de-ionized water, each washing time is also 20mins. Finally, the film was spread on a substrate and dried.

The cross-linked film matrix was placed in ~~the cells of~~a cell culture plate. Immortalized mouse 3T3 fibroblast cell and human fibroblast cell were seeded on the film matrix for ~~the~~ observing~~ation of~~ the growth of cell (~~t~~Please refer to Tables 8,9). The results of cell seeding experiment showed that cell can growth well on the film matrix, and all the cells were alive. ~~while~~ Also, it is stained with neutral red dye. and~~It~~ showed that the film matrix was non-~~cyto~~-toxic~~ity~~ ~~for~~to the human and mouse cell growth.

~~Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made by those skilled in the art without departing from the invention. Accordingly is set out in the following claims.~~

Table 8

~~S~~Example	Seeding of cell No ($x10^4$cell/ml).	1st day ($x10^4$cell/ml)	2nd day ($x10^4$cell/ml)	Third day ($x10^4$cell/ml)
Cross-linked 45C	4	1.8	2.4	4.8
Cross-linked 45D	4	2.4	4.2	7.4
Cross-linked 45E	4	1.4	1.8	3.4

Table 9

~~S~~Example	Seeding of cell No ($x10^4$cell/ml).	1st day ($x10^4$cell/ml)	2nd day ($x10^4$cell/ml)	Third day ($x10^4$cell/ml)
Cross-linked 45C	4	1.2	2.2	5.0
Cross-linked 45D	4	2.6	4.4	7.4
Cross-linked 45E	4	1.6	2.4	4.0

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various

modifications can be made by those skilled in the art without departing from the invention. Accordingly, the scope of the present is limited by the following claims.

A Method for Producing Cross-linked Hyaluronic acid – Protein Bio-composites

Abstract

This invention is concerned with a new method for producing ~~different types of~~ cross-linked hyaluronic acid – protein bio-composites in various shapes. In the present process ~~of manufacture~~, a polysaccharide solution and a protein solution are mixed under moderate pH values ~~and~~in presence of salts to form~~, and the well mixed~~a homogenous solution, which can be process~~epare~~d into various shaprs~~types~~, such as membrane, sponge, fiber, tube or micro-granular and so on. After then, the homogenous solution is subjected to a cross-linking reaction in organic solvent containing weak acid to produce an implantable bio composite material having~~with the~~ excellent bio-compatibility, biodegradability~~tion~~, prolonged enzymatic degradation time, and good physical properties~~of bio-composite is formed in the little acid of organic solution that with the cross-linked reagent~~.

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